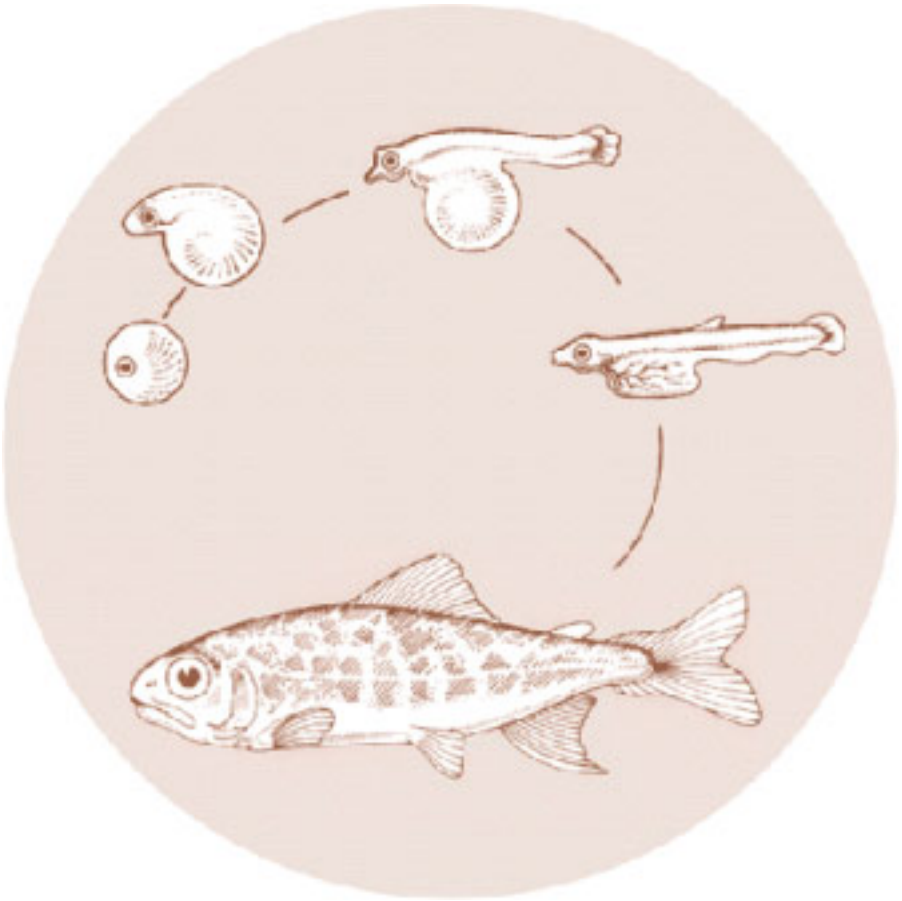
Infrastructure to Complete FDA Registration of Erythromycin

Annual Report
2003 - 2004



DOE/BP-00004544-1

April 2004

This Document should be cited as follows:

Moffitt, Christine, "Infrastructure to Complete FDA Registration of Erythromycin", 2003-2004 Annual Report, Project No. 200000700, 11 electronic pages, (BPA Report DOE/BP-00004544-1)

Bonneville Power Administration
P.O. Box 3621
Portland, OR 97208

This report was funded by the Bonneville Power Administration (BPA), U.S. Department of Energy, as part of BPA's program to protect, mitigate, and enhance fish and wildlife affected by the development and operation of hydroelectric facilities on the Columbia River and its tributaries. The views in this report are the author's and do not necessarily represent the views of BPA.

INFRASTRUCTURE TO COMPLETE FDA REGISTRATION OF ERYTHROMYCIN

Project 2000-007-00

Annual Report for 2003-2004
1 June 2003 – 30 April 2004
Submitted by:

Christine M. Moffitt, PI
Department of Fish and Wildlife Resources
Idaho Cooperative Fish and Wildlife Research Unit
University of Idaho
Moscow, ID 83844-1136
208-885-7047
208-885-9080
cmoffitt@uidaho.edu

28 April 2004

Submitted to:

Ron Morinaka - KEWN-4
Technical Contact

Bonneville Power Administration
US DOE
PO Box 3621
Portland, OR 97208-3621

A) EXECUTIVE SUMMARY OF ACCOMPLISHMENTS:

1. Since accomplishments will not be met without continued funding, we communicated with agency and CBFWA staff to discuss the goals of our research program and risks of lapse in funding.
2. We completed two a trials, one at Lookingglass Hatchery (ODFW) and one at Clearwater Hatchery (IDFG), to evaluate the numbers, diversity and quantify the proportion of isolates resistant to erythromycin before, during, and following administration of erythromycin rations.
3. We completed two trials of bridging methods for detection and quantification of incurred samples of salmon muscle and skin for bridging our microbiological method with HPLC assay of erythromycin residues in salmonid tissues.
4. We interacted with FDA and potential drug company sponsor regarding the manufacturing of the feed additive product.

B) PROGRESS BY OBJECTIVE:

OBJECTIVE 1. Provide an infrastructure to keep erythromycin registration efforts viable in the Columbia River Basin, while required studies are conducted.

Task1.1) Keep all files up to date and maintain archives of all data and communications with FDA.

We completed quarterly reporting and prepared an annual report of releases of fish treated with erythromycin for 2003. We continue to maintain all archives of data, FDA submittals, records of correspondence, and activities of INAD 6013.

Task 1.2) Review and update aspects of the INAD management to make sure that access for erythromycin is maintained during additional approval studies, and during review and negotiations with FDA.

In 2004, we executed production of a new lot of Aquamycin premix. We had inconsistent results testing this product, and elevated potencies were determined. For the past year, we had often documented elevated potencies in finished feed produced at BioOregon. We considered these in error, as the mixing was careful, and all inspections of records showed proper calculations of materials. We had hypothesized that the errors were most likely in the SOPs at the assay laboratory. However, this was a difficult aspect to address, particularly with a lab in another state. We were told following a FDA audit that “Testing to Compliance” with feeds was not good. We spent time working through all phases of the assay process with Denise Moore at Eurofins laboratory. Finally in the past quarter, we determined that Eurofins was extracting samples with residues of the extracting compound remaining. These may have been compounded by standards that could have reduced potency due to hydration after opening. Both these would elevate the estimates of potency for the unknown samples. These assay issues are reasons why FDA wants to have a HPCL method that is a direct not indirect assay of drug potency. On the 16 of April, we released

the premix production from ADM in Des Moines and BioOregon will be producing feeds for the region by the end of April.

Task 1.3) Assist in providing an active dialog with potential drug companies to obtain manufacturing support for this compound after the outstanding details with FDA are resolved.

Paul Rice and a staff member of Bimeda Inc., Bruce Buckmaster of BioOregon, and PI Christine Moffitt participated in a three site Video Conference convened at the University Idaho in December 2003. At that time Bimeda promised to work with BioOregon and U of Idaho to produce of a small lot of feed additive immediately after December holidays at their Le Sueur facility. We sent Bimeda copies of the SOPs for blending the premix product. Unfortunately after this meeting, Bimeda did not quickly pursue this agreement, and in February, they had not begun but came with a proposed price per pound of premix that was more than 40% higher than our incurred costs when we after purchased raw drug from Bimeda, and contracted blending at ADM Animal Health in Des Moines. After discussion with BioOregon and recognition that contract prices for Premix were set in the region, we elected to produce a lot with U of I as manufacturer, using the ADM facilities for contract blending.

OBJECTIVE 2. Conduct laboratory and field experiments and design monitoring to understand the extent of erythromycin resistant microflora in the GI tract of fish following treatment with erythromycin to satisfy the articulated needs of FDA's Division of Human Food Safety, Center for Veterinary Medicine.

Task 2.1. Assess the extent and length of resistant microflora in the GI tract following a normal treatment with erythromycin.

The clinical trial at Lookingglass Hatchery, Oregon Department of Fish and Wildlife, Elgin, OR, was completed in the summer, and the final isolates were identified. In October we began a trial at Clearwater Hatchery, Idaho Department of Fish and Game. Final samples for this trial were obtained in March 2004. Both trials targeted juvenile spring Chinook salmon *Oncorhychus tshawytsha* fed Bio-Oregon rations with Aquamycin 100, but the Lookingglass trial was conducted with 2.25% Aquamycin, fed at 2.2% body weight, and the Clearwater fish were fed 4.5% Aquamycin at 1.2% body weight per day.

The experimental design at Clearwater Hatchery was different from that at Lookingglass Hatchery where all fish were treated. At Clearwater we sampled erythromycin fed fish from two raceways, and compared these with fish from two raceways of fish fed regular rations. In addition, we sampled fish from each of two raceways immediately downstream of the raceways, and one raceway contained fish fed erythromycin (Figure 1). This provided an opportunity to evaluate the differences between fish from first use water and second use water. It also allows for a picture of the effects in fish downstream from erythromycin treated fish.

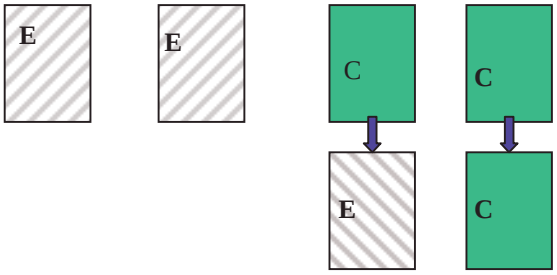


Figure 1. Raceway layout at Clearwater Hatchery. Hatched lines indicates erythromycin fed fish, and the direction of hatch changes for the downstream fed fish. Solid (Green) are the raceways not fed erythromycin (control). Arrows indicate the direction of water flow between raceways.

Methods for sampling fish GI and the general intervals for sampling were similar in each hatchery trial, with more samples obtained from Clearwater hatchery. Fish were sampled before erythromycin treatment began, at two intervals during feeding, and at intervals following feeding. At each sampling, 6 fish were removed randomly from each of raceways and transported live to the University of Idaho. The fish were killed with a blow to the head. The posterior intestine between the last pyloric caeca and the anus was separated, weighed, diluted (w/v) 10-fold in Tryptic Soy Broth (TSB), and pulverized in a sterilized tissue grinder. In the Clearwater trial we used an equalized proportional pooling of homogenates from each fish. The bacterial load was quantified after an 8-d incubation period at 15°C. Colonies were selected at random from plates having 10-100 colonies and transferred to clean agar for pure culture.

Colonies from the posterior intestine have been counted. The number of bacteria isolated from fish at the 26th day of erythromycin treatment at Clearwater Hatchery were summarized for all aerobic bacteria growing on TSA, and for those that grew on TSA with 10 µg/mL (Table 1).

Table 1. Number of aerobic bacterial colonies counted from pulverized tissues from the posterior intestine of spring Chinook salmon (g -1 wet weight ± SE). Counts were made 8 days after plating on TSA or TSA with 10 µg/mL erythromycin. Fish were administered erythromycin rations (110 mg/kg body weight) or a control diet for 26 days at Clearwater Hatchery.

Raceway and Treatment	TSA	TSA & 10 µg/mL Erythromycin
RW 8A-Ery	$6.7 \times 10^{2} \pm 2.0 \times 10^{2}$	$2.7 \times 10^{2} \pm 1.2 \times 10^{2}$
RW 9A Ery	$3.0 \times 10^{2} \pm 1.2 \times 10^{2}$	$2.0 \times 10^{2} \pm 1.2 \times 10^{2}$
RW 10A-Control	$6.5 \times 10^{4} \pm 3.3 \times 10^{2}$	$5.5 \times 10^{4} \pm 5.9 \times 10^{3}$
RW 11A-Control	$1.6 \times 10^{5} \pm 2.0 \times 10^{4}$	$1.1 \times 10^{5} \pm 1.0 \times 10^{4}$
RW 10B-Ery	$1.3 \times 10^{3} \pm 3.2 \times 10^{2}$	$8.0 \times 10^{2} \pm 2.1 \times 10^{2}$
RW 11B-Control	$4.1 \times 10^{4} \pm 3.2 \times 10^{3}$	$3.7 \times 10^{4} \pm 2.0 \times 10^{3}$

Isolates from both agar types were classified by Gram stain, colony morphology, and a sample submitted to Washington Animal Disease Diagnostics Laboratory (WADDL) Washington State University, for identification by biochemical methods using API 20 E and API 20NE kits (bioMerieux, France). We compared the phenotypic identification with

these biochemical identifications and obtained good agreement. Grouping of isolates based on phenotypic characteristics was matched for more than 98% of samples. Use of the API system for fish bacteria is not perfect, but it is increasing in acceptance through documentation (Karatas and Candan 2003). We have selected an array of isolates to profile with genetic techniques, and compare results to biochemical methods. These tests are not completed. More information on sequencing of bacterial isolates and resistance factors is emerging each year and we will utilize these databases and genetic profiles (e.g. Aminov et al. 2002). To date we have tested six isolates. The genetic tools provide for a goodness of fit to the existing sequence database (Table 2).

Table 2. Summary of six isolates identified by API and genetic sequencing.

Fish ID	Bacterial ID (**API**)	Bacterial ID (Molecular Approach)
Fish ID:0412CWPT538A	*Bacillus* sp.	*Bacillus cereus (*>98%)
Fish ID:0412CWPT538A	*Micrococcus* sp.	*Micrococcus psychrophilum* (98%)
Fish ID:0412CWPT5311B	*Aeromonas* sp	Rainbow trout intestinal bacterium (>98%) Chitinibacter tainensis (96%)
Fish ID:0412CWPT5311A	*Pseudomonas fluorescens*	Psychrobacter spp. (>97%)
Fish ID:0412CWPT5311B	*Staphylococcus xylosus*	*Staphylococcus saprophiticus* strain MS510 (98%), *Staphylococcus xylosus* (98%), *Staphylococcus succinus* (98%)
Fish ID:0412CWPT538A	*Aerococcus viridans*	*Aerococcus viridans (>99%)* *Aerococcus* sp. (>99%)

We analyzed samples of medicated and control feeds from Lookingglass, and to date only one lot of erythromycin feed from Clearwater. The Moore-Clarke diet used after treatment at Lookingglass Hatchery had few culturable bacteria, and no erythromycin resistant bacterial colonies were observed. We attribute these differences to differences in the feed manufacturing process. The BioOregon is a cold extrusion process, whereas the Moore-Clark diet is heated and palletized.

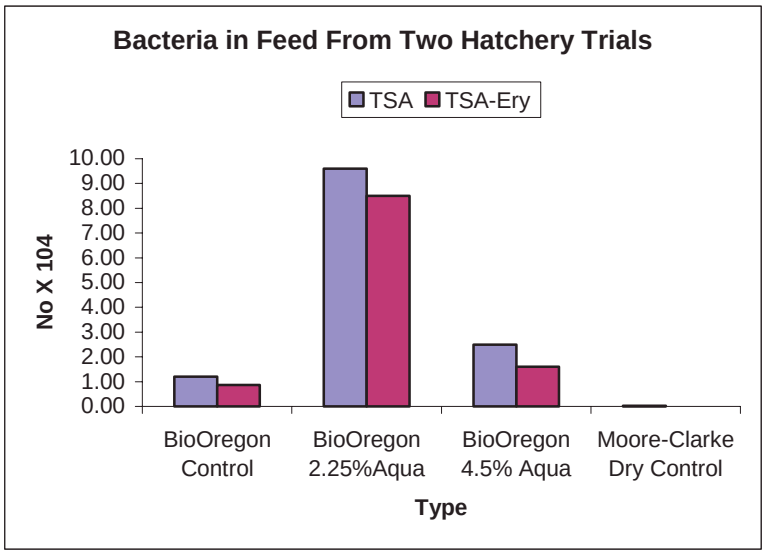


Figure 2. Number of bacteria in the feeds used in trials at Lookingglass (2.25% Aqua) and Clearwater hatcheries (4.5% Aqua). Moore-Clark and Control Diets were used at Lookingglass.

Task 2.2. Assess the extent and length of resistant microflora that remain in the GI tract of fish that remain in freshwater for the duration of their rearing to adult stage.

We will not complete this unless we have continued funding. We will use fish in settling ponds as models and sample at Clearwater Hatchery. Testing of selected resistant isolates will be done using methods recommended by NCCLS (2003) on Mueller-Hinton agar and each strain will be examined for zones of inhibition to selected antibiotics. We will use *Aeromonas salmonicida* as a reference strain as recommended by NCCLS. We will calculate the CFU per gram resulting from each feed type on untreated and treated agars, and compare these with results for relative growth.

OBJECTIVE 3. Conduct experiments to address the fate of erythromycin in sediment ponds with a history of erythromycin treatment.

Task 3.1. Prepare GIS based overviews of regional hatcheries, erythromycin use patterns, flows, salmon stocks and known resident species to determine areas of highest risk and likely sites for monitoring.

We will be completing this aspect in the next project year, if funding is continued.

Task 3.2. Determine the patterns of erythromycin resistance in bacteria isolated from sediment ponds receiving effluents from salmonid rearing systems with a history of erythromycin use, and those without such a history.

We plan to use the sediment ponds at Clearwater for obtaining samples for this task. We plan to involve scientists at the National Center for Toxicological Research (NCTR) in Jefferson, Arkansas, and Dr. Dana Kolpin, USGS, to address these questions. Again, this element is scheduled to begin this summer. We obtained the results of mineralization of erythromycin in sediments provided to the NCTR laboratory. They found that mineralization is rapid, thus the risks of erythromycin remaining in sediments is very low. We will quantify these and provide details in the risk assessments with further sampling from actual trials if funding is continued.

Task 3.3. Determine if the patterns of susceptibility in bacteria isolated from sediment ponds affect the intestinal microflora and their antibiotic susceptibility profiles.

The questions to be addressed is if there is a change in profiles from Task 3.2, does the change in susceptibility in those bacteria isolated from the sediment ponds affect the intestinal micro flora and their antibiotic susceptibility profiles of intestinal micro flora in any freshwater salmonids that live in the environment? Again, if we are not funded in the future, we will not be able to complete these objectives.

OBJECTIVE 4. Conduct studies needed to provide FDA with a satisfactory method of monitoring erythromycin residues in tissues of salmonids.

Task 4.1. Conduct “bridging studies” that show the equivalency of microbiological methods used at the University of Idaho with the proposed regulatory methods developed at FDA’s National Center For Toxicological Research (NCTR).

Throughout this year we participated with FDA in a bridging study of incurred residues of erythromycin in salmonid tissues. In October, Dr. Anita Koehn and Ms. Julie Keniry analyzed two incurred samples sent to us from the FDA lab in Maryland. Both samples were below the limits of detection of our assay, and one produced trace zones. We were able to produce good standard curves for PBS standards and for fortified tissues. We have continued dialog with FDA and have another run scheduled for the second week in March. We will have several conference calls to define the responsibilities and mesh schedules of the key personnel in each of the three labs.

In March, the team assembled again, to conduct bridging tests. We are finalyzing our analysis for submittal to FDA and will complete this within the month of May. We were able to detect potency of the incurred samples sent to us, and in fortified samples that we prepared and sent to Dr. Billideau at NCTR.

OBJECTIVE 4. Provide submittals to FDA that detail results and publish in peer-reviewed journals the results of key studies accomplished during drug approval.

Task 4.1. Summarize the results of studies into complete and comprehensive data submission for review by FDA.

We are still working on the draft antimicrobial resistance assessment under the format in Guidance document 144. This has taken a lot more time than expected, as the format asked for specific methodology. Julie Keniry has been serving as our internal quality assurance officer for the gut microflora project, and has reviewed all SOPs. We still have to complete a section on data reduction methods.

Task 4.2. Continue to submit for publication data submitted to FDA in previously conducted research, clinical and non-clinical trials of erythromycin therapy conducted with Bonneville funding.

The PI submitted one manuscript containing an overview of issues surrounding antibiotic resistant microorganisms in hatchery-reared fish. This will be published in a symposium of Management of Propagates Fishes being prepared by the American Fisheries Society. We continue to work on a more detailed manuscript regarding erythromycin treatment in the Pacific Northwest.

Task 4.3. Provide publication of new trials and assessments conducted in final approval stages.

We have assembled information that can serve as major contributions to the literature on the effects of antibiotic therapy on salmonids. Our new GIS based approaches will be helpful to many parties looking at the more regional impact. We plan on publishing the results of the bridging study.

C) WORK PLANNED FOR NEXT YEAR:

1. Complete genetic analysis of selected isolates from Clearwater Hatchery and Lookingglass Hatchery Trials to determine the agreement of API and genetic profiles.
2. Test selected Gram positive isolates in micro assays to establish the breaking points of resistance.
3. Test selected archives of samples for selected genetic resistance patterns.
4. Continue Quality Assurance aspects of the laboratory trials and field samples.
5. Pursue completing the environmental assessment and bridging study and collaborate with FDA staff to determine who will accomplish which task.
6. Continue to provide feed additive for regional hatcheries.

D) PEER REVIEWED PUBLICATIONS ACCEPTED, PUBLISHED OR SUBMITTED DURING YEAR

A paper is in press. "Evaluating and Understanding Fish Health Risks and their Consequences in Propagated and Free-Ranging Fish Populations**"** This was part of the AFS Symposium held 16-18 June in Boise, entitled Propagated Fish in Resource Management. Authors are: Moffitt, C. M., A. H. Haukenes, and C. J. Williams.

E) ORAL PRESENTATIONS & MEETING ABSTRACTS DURING THE PAST YEAR:

The PI presented an oral paper at the INAD workshop, Bozeman Montana, August 2003. "Progress on drug approval studies of erythromycin to control bacterial kidney disease."

The PI presented an oral paper and a poster at the Northwest Fish Culture Conference, Portland Oregon. December 2003. "Profile of bacteria in the gastrointestinal track of Chinook salmon before during and following administration of erythromycin rations" by Christine M. Moffitt, S. M. Mobin and J. Amberg.

J. Amberg presented an oral paper at the Idaho Chapter AFS in Moscow ID in February 2004. "Profiles of the gut microflora in Chinook salmon fed erythromycin rations"

Post Doctoral Scientist Mobin presented a paper at the Western Division AFS meeting in Salt Lake City, Utah in February 2004.

F) PROBLEMS NEEDING ATTENTION

We have learned that Bonneville recommended a DO NOT FUND, in spite of strong support from regional agencies, CBFWA, and recommendation by the ISRP. The Pacific Northwest Fish Health Protection Committee (PNFHPC) wrote a letter in late September to Bonneville administrators, urging continued support for the project. The Fish and Wildlife Agencies Directors of Idaho, Oregon, Washington sent a letter of support in January 2004 to Mr. Steve Wright of Bonneville Power. NOAA fisheries sent a letter of support to Bonneville in March. The response from Bonneville was still DO NOT FUND, and again agency directors the CBFWA have are drafting an additional letter that specifically addresses obligations for hatchery mitigation and the importance of erythromycin to hatchery management as well as captive brood development. If this project is not continued there is a serious risk for hatchery production and captive broods of Chinook salmon. The PI provides her time to this project without cost to BPA,

but staff that conduct the scientific research and file management cannot continue without funding. Regional agency hatchery coordinators are worried about how lapse of funding will affect their artificial production needs.

All these efforts, and the uncertainty of continued funding are affecting the progress of this project to complete the scientific components that are needed to gain FDA approval.

G) REFERENCES

Aminov, R. I., J. C. Chee-Sanford, N. Garrigues, B. Teferedegne, I. J. Krapac, B. A. White, and R. I. Mackie. 2002. Development, validation, and application of PCR primers for detections of tetracycline efflux genes of gram-negative bacteria. Applied and Environmental Microbiology. 68:1786-1793.

Karatas, S., and Candan, A., 2003. Isolation of *Aeromonas* strains from the intestinal flora of Atlantic salmon (*Salmo salar* L 1758), Turkish Journal of Veterinary and Animal Sciences 27:1071-1075.